IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of
YUMIO KUDO et al.
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Group Art Unit: 1615
Examiner: Kishore, Gollamudi S

For: SYSTEM FOR RELEASE IN LOWER GASTROINTESTINAL TRACT

DECLARATION UNDER 37 C.F.R. 1.132

I, Yumio Kudo, hereby declare and state as follows:

1. I graduated from Hokkaido University, Faculty of Pharmaceutical Science, Department of Physical Chemistry in Hokkaido, Japan in March, 1986. I joined Mochida Pharmaceutical Co., Ltd, the assignee of the present invention of US Patent Application NO. 10/009,265, in April 1986. I currently belong to the Discovery Research I of Dosage Form Creation Pharmaceutical Laboratory in Mochida Pharmaceutical Co., Ltd.

2. Since I joined the company, I have been engaged primarily in the study of manufacturing new and existing drugs in the Pharmaceutical Laboratory of Mochida Pharmaceutical Co., Ltd for 18 years. In particular from 1998 to 2000, I was engaged in the research of a system

for releasing drugs specifically to a large intestine and accomplished the present invention with other joint inventors.

3. The objective of the present invention is to provide a system that unfailingly and quickly delivers an objective material selectively to a lower part of gastrointestinal tract (large intestine) without being influenced by a pH variation in the large intestine or a change in the enterobacterial flora depending on a pathological state and others. The present invention is based on the finding of a system that is capable of releasing a content site-specifically (to the large intestine) by forming a composition comprising a mixture of cystine and chitosan into the form of, for example, a capsule or a coating film.

4. In the Test Example 3 of the original specification of the present application, I verified that the cast film comprising a composition containing cystine and chitosan did not disintegrate in a bicarbonate buffer solution used as a model of a small intestine environment but disintegrated specifically in a suspension of rat cecum contents used as a model of a large intestine environment, and showed that the composition of the present invention is useful as a device for a large intestine-specific

delivery. I also verified that the cast film comprising a composition containing chitosan without cystine did not disintegrate in either test solution. So, this result indicated that the chitosan cast film without cystine could not achieve the same function of large intestine specific delivery.

I have conducted further experimental tests and hereby provide the results thereof to show that there is a fundamental difference between chitosan in combination with cystine in the objective of the present invention, and two other kinds of polymers, i.e., hydroxypropylmethyl cellulose (hereinafter abbreviated as HPMC) as suggested by the Examiner as being easily replaceable, and ethyl cellulose (hereinafter abbreviated as EC) as described in the specification of Iida et al. In the present additional experiments, the tests were conducted for checking the disintegration of cast films each comprising a composition mixed with cystine. The method and the results of the additional experiments are provided below.

5. [Preparation of A Chitosan Film]

After adding 8.4 g of water to 0.6 g of chitosan (Chitosan LL (registered trademark), viscosity (0.5%, 20°C); 20 cps or more, Yaizu Suisankagaku Industry Co.,

Ltd.) and dispersing the chitosan, while stirring the solution, 2.7 g of acetic acid was gradually added to dissolve the chitosan. About 2.5 ml of this solution was spread on a petri dish with the inner diameter of 9.3 cm and dried at room temperature, then humidification treatment (40℃, 75%) was performed for 12 hours to remove the remaining acetic acid, and further dried at room temperature to prepare a cast film.

[Preparation of A Chitosan/Cystine Film]

After adding 6.4 g of water to 0.6 g of chitosan (same as above) and dispersing the chitosan, while stirring the solution, 2.7 g of acetic acid was gradually added to dissolve the chitosan. This solution was added to a suspension that was obtained by dispersing 0.3 g of cystine, which had been atomized by a jet mill, in 2 g of water, and was stirred to obtain a uniformed suspension. About 2.5 ml of this suspension was spread on a petri dish with the inner diameter of 9.3 cm and dried at room temperature, then humidification treatment (40℃, 75%) was performed for 12 hours to remove the remaining acetic acid, and further dried at room temperature to prepare a cast film.

[Preparation of An HPMC/Cystine Film]

After adding 10 g of water to 0.6 g of HPMC

(METOLOSE (registered trademark) 65SH-50, Shin-Etsu Chemical Co., Ltd) and dissolving the HPMC, 10.7 g of ethanol was added. This solution was added to a suspension that was obtained by dispersing 0.3 g of cystine, which had been atomized by a jet mill, in 8.4 g of water, and was stirred to obtain a uniform suspension. About 7 ml of this suspension was spread on a petri dish with the inner diameter of 9.3 cm and dried at room temperature to prepare a cast film.

[Preparation of An EC/Cystine Film]

After adding 10 g of ethanol to 0.6 g of EC (ETHOCEL (registered trademark), Dow Chemical Company) and dissolving the EC, 0.15 g of triethyl citrate was added. This solution was added to a suspension that was obtained by dispersing 0.3 g of cystine, which had been atomized by a jet mill, in 1 g of ethanol, and was stirred to obtain a uniform suspension.

On the side, after adding 5 ml of water to 1 g of gelatin and heating up to 60℃ to dissolve the gelatin, 1 ml of this solution was spread on a petri dish with the inner diameter of 9.3 cm and dried at room temperature. The above uniformed suspension 3 ml was spread over the gelatin film formed on the petri dish and dried at the room temperature to obtain a bilayer film. The bilayer

film was peeled off from the petri dish and it was dipped into the water heated up to 35℃ to dissolve the gelatin. Then, the film was washed with water and dried at the room temperature to obtain a cast film.

[Film Disintegration Test]

The cast films which had been measured their mass in advance were placed and dipped in a sealed vessel containing pH 7.0 bicarbonate buffer (hereinafter abbreviated as BCB) or 33% rat cecum contents suspension (hereinafter abbreviated as C-BCB) which had been obtained by suspending the cecum contents of a Wistar rat with BCB. After purging the head space with carbon dioxide gas, the vessel was sealed and gently shaken for about 16 hours at 37°C. After shaking, if any remnants of the cast films were confirmed, they were taken out. After their external appearance were observed, they were washed with water, photographed and subsequently dried. After drying, the masses of the remnants of cast films were measured and the film mass reduction rates were calculated by the following formula.

The film mass reduction rate (%) = (the film mass before the test - the film mass after the test)/the film mass before the test × 100

In addition, the above test solutions were prepared as follows in view of Tozaki et al. document*1:

BCB:

$NaHCO_3$	9.240 g
$Na_2HPO_4 \cdot 12H_2O$	7.125 g
NaCl	0.470 g
KCl	0.450 g
$CaCl_2 \cdot 2H_2O$	0.073 g
$MgCl_2 \cdot 6H_2O$	0.087 g
H_2O	1 liter.

BCB was prepared by bubbling CO_2 into the solution to adjust it to pH 7.0.

C-BCB:

After extracting a rat cecum, 10 ml of BCB was added per 5 g of cecum contents mass to make a suspension, and subsequently filtered through four-ply gauze to obtain C-BCB.

<Reference Document>

*1: Tozaki H. et al. : Metabolism of peptide drugs by the microorganisms in rat cecal contents Biol. Pharm. Bull. 18(6) 929-931 (1995)

The film disintegration test results:

Film	Test solution	Film mass reduction rate (%)	External appearance	Disintegration [1)]
Chitosan	BCB	24.6	No Change	No
	C-BCB	52.7	Turned brown, and slightly thinner	No
Chitosan+ Cystine	BCB	26.9	No Change	**No**
	C-BCB	89.9	Turned black and fragmented[3)]	**Yes**
HPMC+ Cystine	BCB	— [2)]	Dissolved immediately after the test had started	Yes
	C-BCB	— [2)]	Dissolved immediately after the test had started	Yes
EC + Cystine	BCB	16.2	No Change	No
	C-BCB	24.3	No Change	No

1) Yes: Film disintegration (collapse) or film decomposition

No: Film remains in its original form

2) There were no remnants of the cast film, thus the film mass reduction rate was not calculated.

3) Turned black and fragmented: The results showed very similar results to the photograph of Fig.6 (a) in the original specification (Specification Example 5, chitosan/Eudragit E100/cystine). The result shown in this photograph of Fig.6 may cause confusion at a glance, thus a diagrammatic illustration of the figure is attached for easier understanding. Also, the photograph

of Fig.6 (b) in the original specification is the result of the Comparative Example 2 of the specification and shows that the film appears to be white and not dissolved in the model solution of a small intestine and the film appears to be black but not dissolved in the cecum contents suspension, in comparison to the result of Fig.6 (a).

6. In the film disintegration test, the cast film comprising a mixture of a hydrophilic polymer, i.e. HPMC and cystine dissolved in both BCB and C-BCB immediately after the test had started, therefore, the cast film could not be used as a protective coating from small intestine juice. Even if an enteric coating was further applied over the preparation comprising this composition and the preparation might successfully pass through the stomach, it would be dissolved in the small intestine immediately and unable to deliver the contents to the large intestine.

On the other hand, in the case of the cast film comprising a mixture of a water insoluble polymer, i.e. ethyl cellulose (EC) and cystine, there was no disintegration in either BCB or C-BCB and the result suggested that the preparation comprising this

composition would not be capable of releasing an active ingredient even after reaching the large intestine.

On the contrary, in the case of the cast film comprising a mixture of cystine and chitosan, as indicated in Test Example 3 of the specification, the disintegration did not occur in BCB but disintegration was identified in C-BCB. That is, when cystine was combined with chitosan, it showed that the preparation comprising this composition does not disintegrate in the small intestine but disintegrates specifically in the large intestine and releases an active ingredient. Further, it can be seen that the disintegration of these cast film does not necessarily depend on pH of the site where the disintegration occurs (it is not necessary as an essential feature although it is preferable that pH would be lower in the large intestine).

Based on the foregoing results, it is clear that the composition of the present invention comprising a mixture of cystine and chitosan has an excellent function of unfailing and quick disintegration selectively in the lower part of the gastrointestinal tract (large intestine) without being influenced by a pH variation in the intestine or a change in the enterobacterial flora depending on a pathological state and others. The

objective of the chitosan is not equivalent to that of a polymer (e.g. HPMC, EC) suggested by the examples in the prior art (Iida et al.) and therefore there is no motivation to make a replacement of their components.

FIG. 6(a)

A diagrammatic illustration of FIG. 6(a)

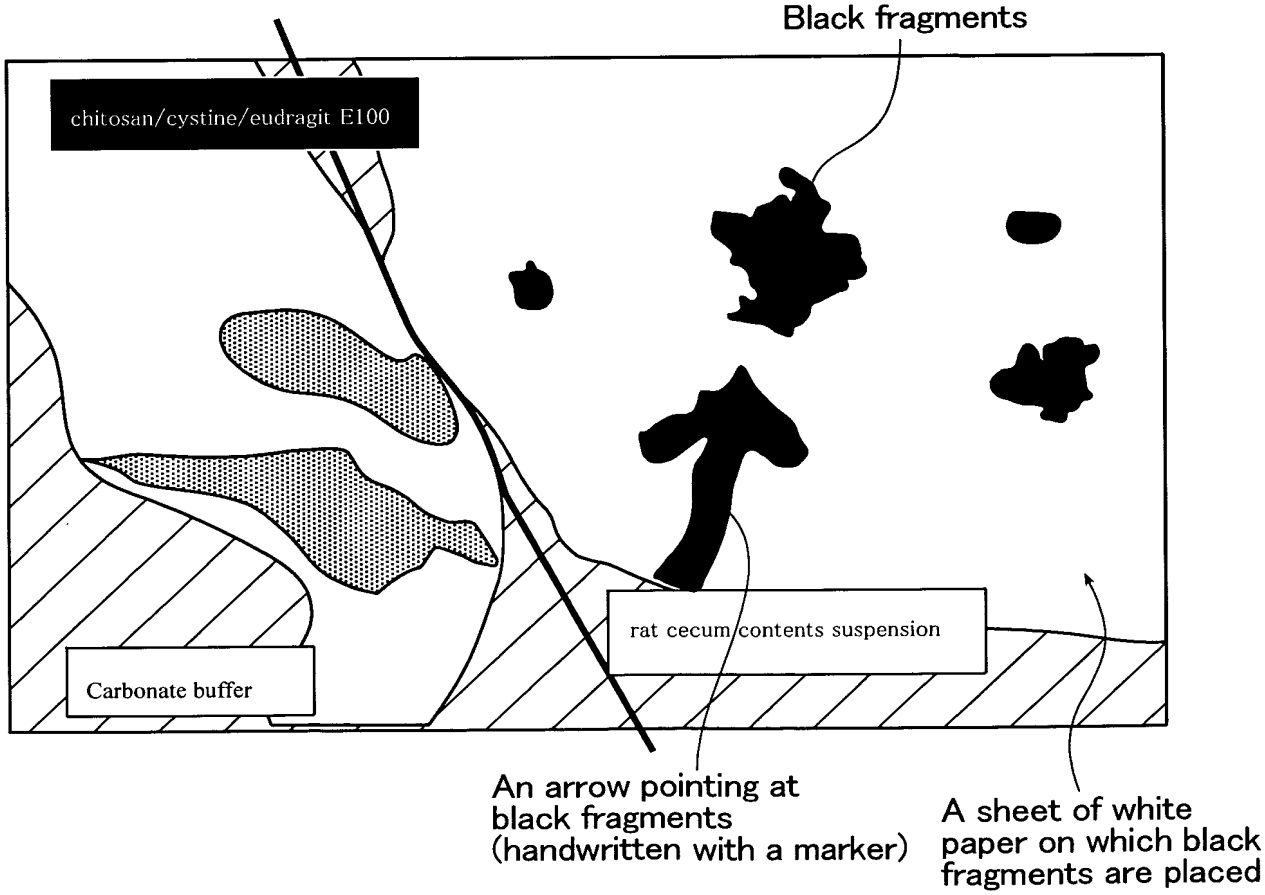
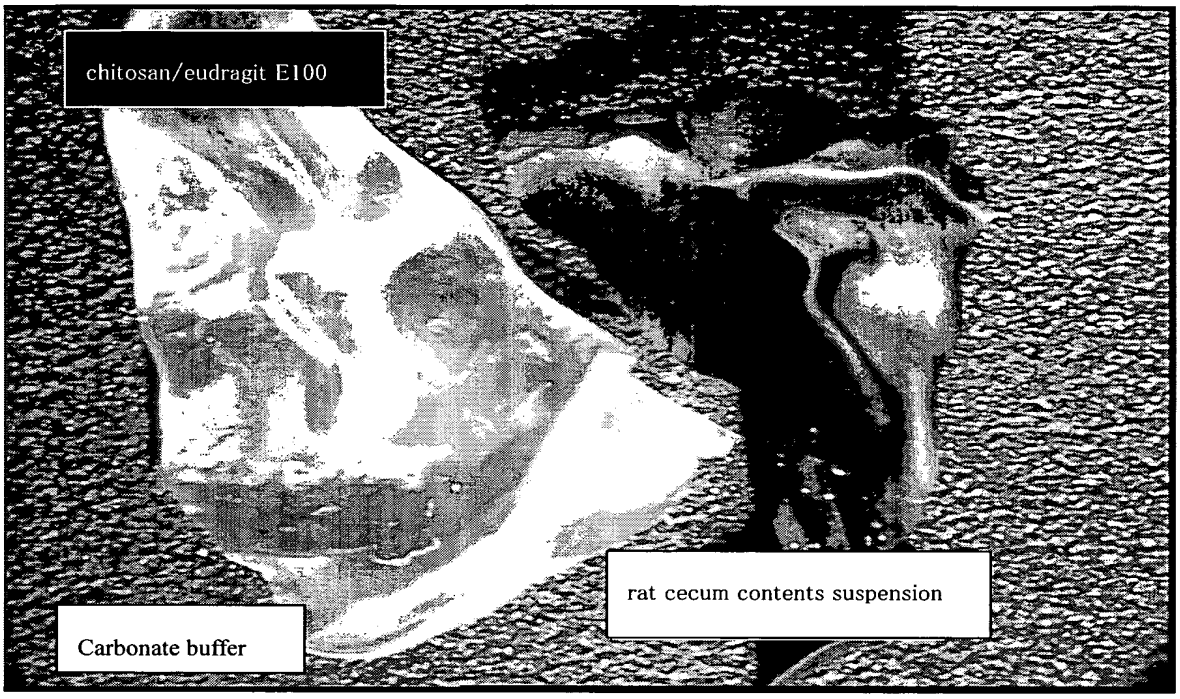


FIG. 6(b)



I declare that all statements made herein of my own knowledge are true and that all statements made on my own information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like might be punishable under the applicable law or may jeopardize the validity of the application or any patent issuing thereon.

Yumio Kudo

Yumio KUDO

December 14, 2004

Date

METABOLISM OF PEPTIDE DRUGS BY THE MICROORGANISMS IN RAT CECAL CONTENTS

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The metabolism of insulin and calcitonin by microorganisms was examined in rat cecal contents. Both insulin and calcitonin were markedly degraded. Calcitonin was more susceptible to proteolysis in rat cecal contents than insulin. Calcitonin was also rapidly degraded in supernatant, while we found few degradation products of insulin. These findings suggest that care should be taken to metabolize the peptide drugs by microorganisms when they are administered to the large intestine for colon-specific drug delivery.

KEY WORDS insulin; calcitonin; microorganism; colon-specific drug delivery

It is well known that the activities of various proteases which are responsible for peptide degradation in the large intestine are generally lower than in the small intestine.[1-4] Therefore, many studies have investigated the delivery of peptide and protein drugs to the colon.[8-11]

On the other hand, various microorganisms are distributed throughout the gastrointestinal tract, and most of these are found in the large intestine where they mediate hydrolytic digestive functions using carbohydrate and proteins as substrates. In addition, these microorganisms have the potential to metabolize drugs and other foreign compounds including peptide drugs. However, there have been very few studies concerning the metabolism of peptides in the large intestine by these microorganisms.[7] Therefore, in this study, insulin and calcitonin were chosen as model peptides, and the metabolic characteristics of these peptides by the microorganisms were examined in rat cecal contents.

MATERIALS AND METHODS

Preparation of Drug Solutions

Bovine insulin (Sigma, Chemical Co., St. Louis, MO, USA) and human calcitonin (SUNTORY, Osaka, Japan) were dissolved in isotonic phosphate buffer at pH 7.4 to yield the final concentration of 0.1 mM.

Preparation of Cecal Contents Suspension and Its Supernatant

Fresh cecal contents from non-fasted rats were suspended in two-fold their volume of bicarbonate buffer ($NaHCO_3$, 9.240 g ; $Na_2HPO_4 \cdot 12H_2O$, 7.125 g ; NaCl, 0.470 g ; KCl, 0.450 g ; $CaCl_2 \cdot 2H_2O$, 0.073 g ; $MgCl_2 \cdot 6H_2O$, 0.087 g/l). The pH of the buffer was adjusted to 7.0 by bubbling with CO_2 gas prior to use. The suspension was filtered through four layers of gauze. Supernatant was obtained by centrifuging the cecal suspension at 3,000 rpm for 5 min.[7]

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In Vitro Stability Experiments

The degradation of insulin and human calcitonin in 33% suspension and supernatant of rat cecal contents was studied by incubating 0.1 mM insulin or calcitonin solution with 33% suspension or supernatant of rat cecal contents. Samples were withdrawn from the incubation mixture, and 50% acetic acid was added to terminate the reaction. Then the resulting mixture was centrifuged for 5 min to remove the precipitated protein and cecal contents. Twenty-five microliters of the supernatant were injected into HPLC.[7]

RESULTS AND DISCUSSION

The concentration-time profiles for the degradation of insulin and calcitonin from the rat cecal contents are shown in Fig. 1. Both insulin and calcitonin were metabolized in 33% suspension of rat cecal contents, but the degradation of calcitonin was much faster than that of insulin.

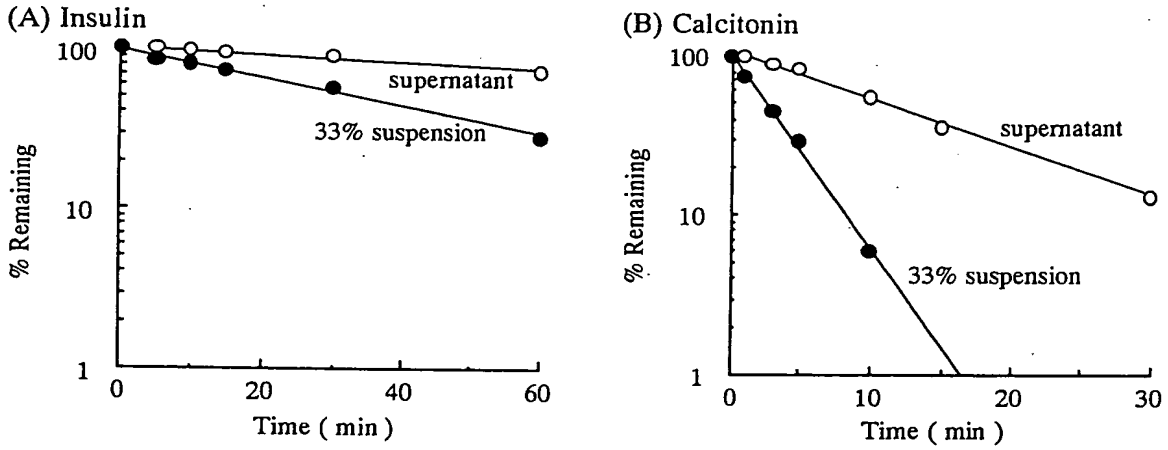

Fig. 1. Degradation Profiles of (A) Insulin and (B) Calcitonin in Rat Cecal Contents Results are expressed as the mean ± S.D. of 3 experiments. Keys: ● , 33% suspension of rat cecal contents; ○ , supernatant of 33% suspension of rat cecal contents.

Table 1 shows the degradation half-lives of insulin and calcitonin in 33% suspension of rat cecal contents. The half-life for the proteolysis of calcitonin in 33% suspension of rat cecal contents was 13-fold smaller than that of insulin. These findings suggested that insulin and calcitonin might be degraded by some peptidases of microorganisms in rat cecal contents as well as proteolytic enzymes in the intestinal homogenates and intestinal fluid of rats.[3-6] Furthermore, it was suggested that microorganisms in rat cecal contents might contain some proteolytic enzymes which are responsible for calcitonin hydrolysis rather than insulin degradation.

Table 1. Degradation Half-Lives of 0.1 mM Insulin and Calcitonin in Rat Cecal Contents

	Insulin (min)	Calcitonin (min)
33% suspension	33.6 ± 5.8	2.5 ± 0.3
Supernatant	125.9 ± 3.4	9.8 ± 0.4

Results are expressed as the mean ± S.D. of 3 experiments.

We also examined the metabolism of these peptides in supernatant of 33% suspension of rat cecal contents. As shown in Fig. 1, calcitonin was rapidly degraded in supernatant of rat cecal contents: less than 20% of calcitonin remained after 30 min. In contrast, insulin was almost stable in such supernatant of rat cecal contents. Table 1 shows the degradation half-lives of insulin and calcitonin in supernatant of 33% suspension of rat cecal contents. The half-life for the proteolysis of calcitonin in this supernatant was 13-fold smaller than that of insulin. This finding indicated that calcitonin was metabolized by both enzymes released from the microorganisms and their membrane enzymes in rat cecal contents. On the other hand, insulin was mainly metabolized by the membrane enzymes of the microorganisms.

We have not determined the proteolytic enzyme types of these microorganisms in rat cecal contents. However, in our pilot studies, it may be considered that there exist aminopeptidases, trypsin, and chymotrypsin-like peptidases in the microorganisms in rat cecal contents, since the degradation of insulin was inhibited by camostat mesilate, aprotinin, and soybean trypsin inhibitor in rat cecal contents. We are now determining the types of proteolytic enzymes and the effects of various protease inhibitors on the metabolism of these peptides in rat cecal contents, and these results will be reported in a subsequent paper.

In conclusion, the present study indicated that peptide drugs such as insulin and calcitonin were degraded by the microorganisms in rat cecal contents. The metabolism of peptide drugs by the microorganism in the large intestine should be taken into account, when colon-specific peptide drug delivery systems are designed.

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